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Putative Molt-Inhibiting Hormone in Larvae of the Shore Crab *Carcinus maenas* L.: An Immunocytochemical Approach

S. G. WEBSTER[1] AND H. DIRCKSEN*

*School of Biological Sciences, University College of North Wales, Bangor, Gwynedd LL57 2UW, UK, and *Institut für Zoophysiologie, Universität Bonn, Endenicher Allee 11-13, D-5300 Bonn 1, Germany.*

Abstract. Immunocytochemical investigations of the eyestalk of *Carcinus maenas* zoeal larval stages, using an antiserum directed against putative *Carcinus* molt-inhibiting hormone (MIH), revealed immunopositive neuronal structures. These structures included perikarya associated with the medulla terminalis X-organ, parts of the sinus gland tract, and the neurohemal organ—the sinus gland. Apart from an increase in volume of the sinus gland between zoeal stage I and II, no striking changes in the topography or morphology of the MIH neurosecretory system were observed. Immunopositive structures were found in similar locations to those seen in adult crabs. Our results suggest that the control of molting by MIH in crustacean larvae may be similar to the currently accepted model of molt control in adult decapod crustaceans.

Introduction

A current model of molt control in decapod crustaceans involves regulation of ecdysteroid synthesis by a molt-inhibiting hormone (MIH), released by neurosecretory neurons in the eyestalk. Much evidence has now accumulated suggesting that increased synthesis and titers of circulating ecdysteroids necessary for induction of premolt are directly repressed by this neuropeptide, thus inhibiting proecdysis and molting. Nevertheless, alternative hypotheses have implicated processes such as metabolism and excretion of ecdysteroids in molt regulation (see Skinner, 1985; Webster and Keller, 1988; Watson *et al.*, 1989 for recent reviews). Despite recent advances in our knowledge concerning mechanisms of molt control in adult decapod crustaceans, little is known about the regulation of molting in larval crustaceans. This deficiency has been reiterated in a recent review by Christiansen (1988).

Evidence for molt regulation by MIH in crustacean larvae has, until recently, been obtained by eyestalk ablation experiments (for references see Charmantier *et al.*, 1988; Christiansen 1988), which have given equivocal results, suggesting that in some instances, the larval molt is not regulated by MIH until shortly before metamorphosis. However, with regard to morphological correlates of neurosecretory structures in larval eyestalks, several reports (Orlamünder, 1942; Pyle, 1943; Hubschman, 1953; Dahl, 1957; Matsumoto, 1958; Little, 1969; Zielhorst and Van Herp, 1976; Bellon-Humbert *et al.*, 1978; Gorgels-Kallen and Meij, 1985) detail the ontogeny of larval neurosecretory systems in a wide variety of crustaceans. With the exception of studies by Gorgels-Kallen and Meij (1985), Beltz and Kravitz (1987), and Beltz *et al.*, (1990), there are no other studies in which neurosecretory systems containing immunocytochemically defined neuropeptides have been described in crustacean larvae.

Recently, we have characterized a neuropeptide from the sinus gland of *Carcinus maenas*, which, by virtue of its ability to repress ecdysteroidogenesis by Y-organs cultured *in vitro*, could be described as a *putative* MIH (Webster, 1986; Webster and Keller, 1986). It should be stressed that the precise significance and function of this neuropeptide as a molt-inhibitor *in vivo* has not yet been elucidated, and until suitable *in vivo* bioassays are developed, the status of MIH must remain "putative." Re-

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[1] To whom correspondence should be sent.

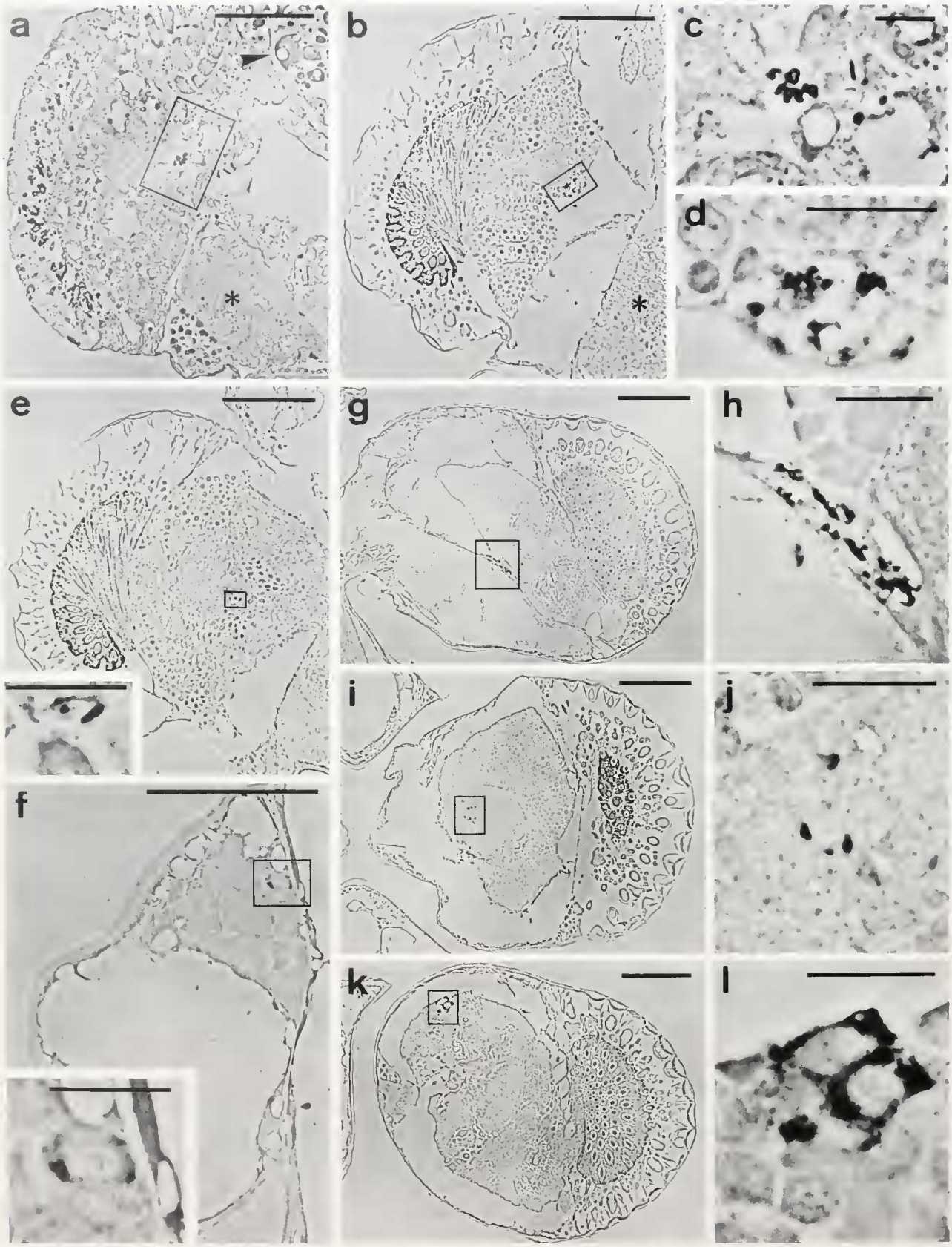

Figure 1. Characteristic structures of MIH-immunoreactive (IR) neurons in prezoeal (a, c), stage I zoea (b, d–f, left eye), and stage II zoea (g–l) eyes of *Carcinus maenas* larvae. Phase contrast micrographs of immunostained semithin (1 μm) transverse sections. (Orientation of dorsal parts of larvae to the tops of micrographs.)

cently, we demonstrated that the neurosecretory system produced putative MIH in the eyestalk ganglia of several adult brachyuran crustaceans (Dircksen *et al.*, 1988). Because these studies provide compelling evidence to suggest that MIH is a secretable neuropeptide, and in view of our earlier observations on the nature and mode of action of this neuropeptide on ecdysteroid synthesis in *Carcinus* (Webster and Keller, 1986; Lachaise *et al.*, 1989), it seemed opportune to examine the larval eyestalk neurosecretory system immunocytochemically, using antibodies raised against *Carcinus* MIH. Evidence presented here suggests that a functional MIH-like neurosecretory system exists in all larval stages of *Carcinus*.

Materials and Methods

Laboratory rearing of larvae

Ovigerous *Carcinus maenas* L. females were collected from the Menai Strait, North Wales, between May and July, and maintained in the laboratory until larvae were released. Only positively phototropic, rapidly swimming larvae were collected. Rearing techniques were initially based upon those of Rice and Ingle (1975), but were found to be inadequate. Successful rearing to first crab with a high survival was achieved using a mixed diet of (A) phytoplankton (*Tetraselmis chuii*), (B) rotifers (*Brachionus plicatilis*), (C) barnacle nauplii (*Elminius modestus*), and (D) brine shrimp nauplii (*Artemia salina*). During each larval stage, prey ratios were supplied as follows: Zoea I (A):1, (B):1, (C):1. Zoea II (A):1, (B):1, (C):1, (D):1. Zoea III (C):1, (D):1. Zoea IV, Megalopa and First crab (D):1. With the exception of phytoplankton (culture density *ca.* 10^6 cells ml^{-1}: 1 part = 15 ml), the total prey concentration was around 25–50 items per ml. Larvae were reared in 50-ml plastic containers in constantly aerated, filtered seawater (33‰) under ambient temperature (15–18°C) and photoperiod (L 15–18 h: D 9–6 h). Maximum density of larvae was 1 per 5 ml. Water and food were changed every two days, at which time instars were staged according to Rice and Ingle (1975). Under these maintenance conditions, survival was good (80%), and instar durations were approximately: Z I: 7, Z II: 5, Z III: 6, Z IV: 7, M: 8, days. Samples of larvae were taken at the middle of each instar, which was considered to be during intermoult.

Tissue processing and immunocytochemistry

Fixations were carried out in a mixture of 2% paraformaldehyde, 2% glutaraldehyde, and 0.1% saturated picric acid in 0.1 *M* sodium cacodylate buffer, pH 7.4, supplemented with 0.5 *M* sucrose and 5 m*M* $CaCl_2$ for 2–4 h at 4°C according to Dircksen *et al.* (1987). Tissues were washed extensively in the same buffer, dehydrated, and embedded in low viscosity resin (Spurr, 1969). Semithin frontal cross-sections (1 μm) through the whole animal were cut on a LKB Ultrotome III or a Reichert Ultracut E, and processed for immunocytochemistry using a rabbit antiserum (code R1TB) directed against HPLC-purified MIH of *Carcinus* (Dircksen *et al.*, 1988), diluted 1:4000 in 0.01 *M* phosphate buffered saline (PBS) and PAP staining techniques (Dircksen *et al.*, 1987). Micrographs were taken with a Zeiss Axioskop using phase contrast optics and documented on Agfapan 25 film.

Results

Despite several attempts to improve the penetration of fixative into the eyestalks (for example, by piercing the exoskeleton behind the cyestalks, using other fixatives or fixation times), adequate fixation of megalopae and first crab stages was impossible. Thus, by necessity, this study is restricted to the zoeal stages of *Carcinus*, and in later zoeal stages problems with fixation and tissue shrinkage were encountered. A sometimes confusing feature of the zoeal eyestalk was the presence of a pigmented perineural sheath (Fig. 2c, 2f), which could have been identified as an immunopositive structure. This problem was resolved by using normal bright field optics, under which immunopositive material appears brownish, or by higher mag-

(a) MIH-IR axon profiles within the sinus gland (center of *rectangle*) of a prezoea. Note ommatidial primordia, brain (*) and yolk droplets (*arrowhead*). (b) MIH-IR axon profiles within the sinus gland (*rectangle*) of a stage I zoea. Note dense pigmentation at the base of the ommatidia, and well-developed neuropiles of the lamina ganglionaris (LG), medulla externa (ME), and the brain (*). (c, d) Higher magnifications of sinus glands corresponding to *rectangles* in a, b. (e) Cross-sectioned MIH-IR axons (inset enlarged from the *rectangle*). (f) Two MIH-IR perikarya in an anterior dorsal cell group of the left eyestalk ganglia (inset enlarged from the *rectangle*). (g) MIH-IR axon profiles in the sinus gland (*rectangle*) of a stage II zoea adjacent to the ME and large hemolymph spaces. Note stalk formation of the eye at this stage. (i) Cross-sectioned MIH-IR axons in the medulla terminalis. (k) Three clustered MIH-IR perikarya in an anterior dorsal position of the presumptive X-organ cell group. Note well-developed ganglia and neuropiles in the eye. (h, j, l) Higher magnifications of *rectangles* outlined in g, i, k. Note axon profiles and putative terminals abutting on the surface of the sinus gland (h) and dark PAP reaction products restricted to the cytoplasm of the perikarya (l) of MIH-IR neurons.

Scale bars: 50 μm in a, b, e, f, g, i, k. 10 μm in c, d, h, j, l, and insets in e, f.

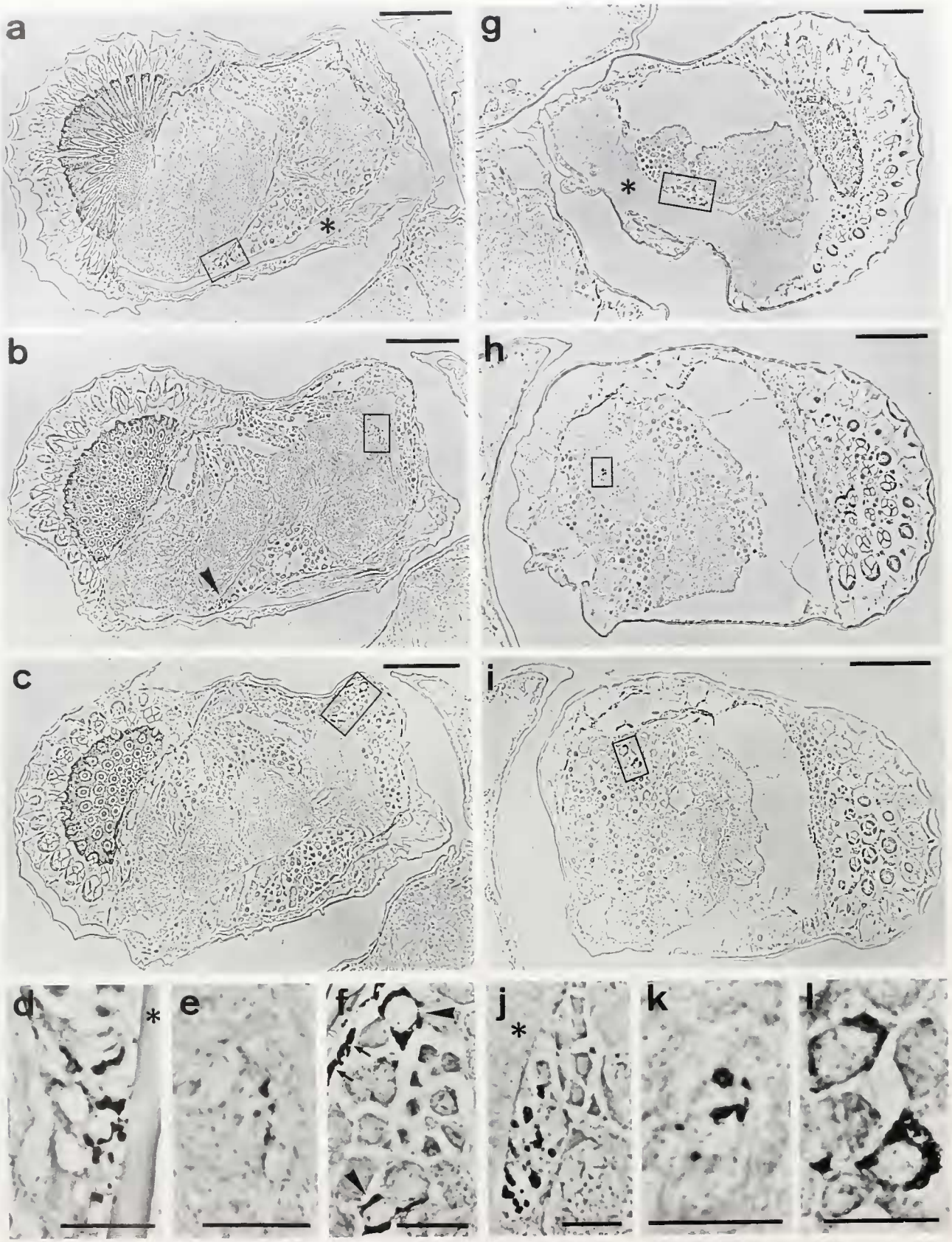

Figure 2. Characteristic structures of MIH-immunoreactive (IR) neurons in stage III zoea (a–f, left eye) and stage IV zoea (g–l, right eye) eyes of *Carcinus maenas* larvae. Phase contrast micrographs of immunostained semithin (1 μm) transverse sections. (Orientation of dorsal parts of the larvae to the tops of the micrographs.)

nification (Fig. 2f) when the black pigment granules could be clearly resolved by phase contrast optics.

MIH immunoreactivity was found in all zoeal stages examined, including the so-called prezoeal stage, which, in view of its brevity (*ca.* 30 min), and association with hatching, could well be described as an embryonic molt. (Fig. 1a, c). In general, MIH immunoreactivity was found in structures similar to those found in the adult, including perikarya in a position similar to the X-organ in adults, an X-organ sinus gland tract, and a sinus gland (Figs. 1, 2). In several preparations the sinus gland appeared to be in close proximity to a large hemolymph vessel (Figs. 1h, 2a, d). By serially sectioning through the entire eyestalk, a maximum of four immunopositive perikarya of about 8–10 μm in diameter were observed in all zoeal stages localized in a cluster of neuroblasts in an anterior dorsal position of the eyestalk, with large nuclei and scarce cytoplasm (Figs. 1f, k, l. 2c, f, i). Axonal projections were found in the medulla terminalis of the well-developed eyestalk ganglia in a typical circular arrangement of four cross-sectioned axons (Figs. 1j, 2e, k), reminiscent of the axonal arrangement in the adult crab. This pattern was found in all zoeal stages. Despite exhaustive investigation, the only discernable change in the morphology of the neurosecretory structures was the size of the sinus gland, which appeared to increase in volume between zoea I and II, when the eye became stalked and mobile. Indeed, it was frequently difficult to observe the sinus gland in zoea I due to its small size, but in zoea II, the sinus gland was often the most striking immunopositive structure (Fig. 1b, d, g, h). In control incubations, preabsorbtion of the antiserum with 2 nmoles of MIH per μl of crude antiserum completely abolished immunostaining, thus proving the specificity of the immunocytochemical detection (results not shown).

Discussion

In the present study, the location of perikarya, axons, and sinus gland terminals immunopositive for MIH have been demonstrated in all zoeal instars of *Carcinus* larvae. Surprisingly, larval immunopositive structures were topographically and morphologically similar to those found in the adult crab. However, very few (maximum 4) MIH-immunoreactive perikarya were observed in any larval stage, compared to the adult crab where there are 32–36 MIH-immunoreactive perikarya (Dircksen *et al.*, 1988). It is likely that the increase in number of immunopositive cells during larval to juvenile/adult development is due to increased MIH gene expression rather than by cell division because neuroblasts are generally considered to be too highly differentiated to undergo further division. A striking similarity of the larval MIH immunopositive structures to those of the adult concerns the morphology of the X-organ sinus gland tract. In the adult, MIH immunoreactive axons form a peripheral tract around the central axon bundle containing crustacean hyperglycemic hormone (CHH) immunopositive axons (Dircksen *et al.*, 1988). Although we did not determine CHH in the present study, the similarity in the arrangement of the four MIH-immunoreactive axons around a central tract was clearly suggestive of the adult morphology.

Several studies have reported the general development of neural systems in the crustacean eyestalk. Cells corresponding to the X-organ have been found in the first larval stages of all species examined (*Birgus*, Orlamünder, 1942; *Homarus, Pinnotheres*, Pyle, 1943; *Crangon*, Dahl, 1957; *Potamon*, Matsumoto, 1958; *Palaemonetes*, Hubschman, 1963; *Palaemon*, Little, 1969, Bellon-Humbert *et al.*, 1978; *Astacus*, Zielhorst and Van Herp, 1976, Gorgels-Kallen and Meij, 1985). With regard to the development of the sinus gland, for freshwater crustaceans, which hatch at an advanced developmental stage, the sinus gland is present in the first larval stage (Matsumoto, 1958; Gorgels-Kallen and Meij, 1985). In marine crustaceans, which hatch at a relatively early stage of development, and which often undergo a lengthy planktonic existence prior to a dramatic metamorphosis, all studies suggest that the sinus gland develops (or can first be observed) late in larval life, at about the time

(a) MIH-IR axon profiles in the sinus gland (*rectangle*) adjacent to the large hemolymph vessel (*) of the eyestalk. (b) Section slightly anterior to (a) showing the sinus gland (*arrowhead*) and cross-sectioned MIH-IR axons (*rectangle*) in the medulla terminalis. (c) Four MIH-IR perikarya (*rectangle*) are found in an anterior dorsal position of the presumptive X-organ cell group. (d, e, f) Higher magnifications of *rectangles* outlined in a, b, c. MIH-IR putative axon terminals adjacent to the hemolymph vessel (*) are found in the sinus gland (d). Note also cross-sectioned MIH-IR axons (e) in the medulla terminalis and strong immunoreactivity of three perikarya (f, *arrowheads*). *Arrows* in (f) point to dark pigments usually found in perineural sheaths of eyestalk ganglia. (g) MIH-IR axon profiles in the sinus gland (*rectangle*) adjacent to the large hemolymph vessel (*) of the eyestalk. (h) Cross-sectioned axons of the presumptive X-organ sinus gland (XO-SG) tract in the medulla terminalis. (i) Two MIH-IR perikarya in the presumptive X-organ cell group in a dorsal anterior position of the proximal eyestalk ganglia. (j, k, l) Higher magnification of *rectangles* outlined in g, h, i, MIH-IR axon profiles and putative axon terminals abutting on the surface of the sinus gland, (*) indicates hemolymph vessel. (j), MIH-IR axons in the XO-SG tract (k) and two strongly immunopositive XO perikarya (l). Note unstained axons in the center of the XO-SG tract (k).

Scale bars: 50 μm in a–c, g–i. 10 μm in d–f, j–l.

of metamorphosis (stage V *Palaemonetes,* Hubschman, 1963, *Palaemon,* Bellon-Humbert *et al.,* 1978; stage III *Homarus,* Pyle 1943). Apart from a report by Jaques (1975) demonstrating the presence of a sinus gland in stage 1 *Squilla mantis* larvae, this paper reports the first demonstration of a sinus gland in first stage larvae of a marine decapod crustacean, and is undoubtedly due to the great resolving power of immunocytochemical techniques compared to conventional histochemical staining methods. To our knowledge, the only other reports using immunocytochemical techniques to identify larval neurosecretory structures are those by Gorgels-Kallen and Meij (1985), demonstrating the neurosecretory structures containing CHH immunoreactivity in *Astacus leptodactylus* larvae, and Beltz and Kravitz (1987) and Beltz *et al.* (1990), demonstrating proctolin-like immunoreactivity in the CNS of larval *Homarus americanus.*

While immunocytochemical evidence indicates that *Carcinus* zoeae possess a MIH neurosecretory system, which may participate in the control of larval molting, experiments involving eyestalk ablation in several species of crustacean larvae (see specific examples in Charmantier *et al.,* 1988; Christiansen, 1988) have demonstrated that, in general, eyestalk ablation is only effective in accelerating proecdysis and molting when performed during the last instar before metamorphosis. Although the deficiencies of these experiments have been commented upon by Freeman and Costlow (1980), particularly with regard to difficulties in determining the precise duration of instars and the time of initiation of proecdysis in rapidly moulting larvae, it has been suggested (Freeman *et al.,* 1983) that the larval molt cycle is not regulated by MIH until metamorphosis. However, studies demonstrating that larval ecdysteroid titers cycle in a molt-stage-dependent manner in much the same way as adults (Chang and Bruce, 1981; Spindler and Anger, 1986), and a report by Snyder and Chang (1986), demonstrating that increases in proecdysial ecdysteroid titer induced by eyestalk removal of Stage II *Homarus* zoeae can be repressed by the injection of adult sinus gland extracts, strongly support the hypothesis that larval molting (or at least, initiation of proecdysis) is regulated by MIH, and the results presented here would also support this hypothesis. However, it should be stressed that no firm inferences as to the function of the immunoreactive MIH can yet be made; it is not known whether larval MIH-immunoreactive material is identical to that in adults, although the antiserum used displays a very high specificity in immunodot assays (Dircksen *et al.,* 1988), RIA, and ELISA (Webster, unpub.), or whether it is released during the zoeal stages. Although *in vivo* experiments involving injection of MIH or sinus gland extracts into zoeal larvae and subsequent monitoring of proecdysis or instar length would undoubtedly strengthen hypotheses concerning larval molt control, the small size of most crab zoeae argues against the success of such experiments in crab larvae. A further problem, which remains unresolved, concerns the increase in number of immunoreactive perikarya between the last zoeal stage and the adult. It is possible that this transition occurs during metamorphosis (a phenomenon we could not elucidate due to difficulties in achieving adequate fixation of megalopae and first crab stages). If the MIH secretory system became synthetically active at this time, and stored MIH was released, then previous observations regarding the failure to accelerate molting in zoeal larvae, and the appearance of the sinus gland as a structure stainable by conventional histochemical methods prior to metamorphosis, could be reconciled with the model of molt control suggested by Freeman *et al.* (1983).

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